Preclinical Development

Targeting the Microtubular Network as a New Antimyeloma Strategy
Rentian Feng1, Shirong Li1, Caisheng Lu1, Carrie Andreas1, Donna B. Stolz2, Markus Y. Mapara1, and Suzanne Lentzsch1

Abstract
We identified nocodazole as a potent antimyeloma drug from a drug screening library provided by the Multiple Myeloma Research Foundation. Nocodazole is a benzimidazole that was originally categorized as a broad-spectrum anthelmintic drug with antineoplastic properties. We found that nocodazole inhibited growth and induced apoptosis of primary and multiresistant multiple myeloma cells cultured alone and in the presence of bone marrow stromal cells. Nocodazole caused cell-cycle prophase and prometaphase arrest accompanied by microtubular network disarray. Signaling studies indicated that increased expression of Bim protein and reduced X-linked inhibitor of apoptosis protein and Mcl-1 levels were involved in nocodazole-induced apoptosis. Further investigation showed Bcl-2 phosphorylation as a critical mediator of cell death, triggered by the activation of c-jun-NH₂ kinase (JNK) instead of p38 kinase or extracellular signal-regulated kinases. Treatment with JNK inhibitor decreased Bcl-2 phosphorylation and subsequently reduced nocodazole-induced cell death. Nocodazole combined with dexamethasone significantly inhibited myeloma tumor growth and prolonged survival in a human xenograft mouse model. Our studies show that nocodazole has potent antimyeloma activity and that targeting the microtubular network might be a promising new treatment approach for multiple myeloma. Mol Cancer Ther; 10(10); 1886–96. ©2011 AACR.

Introduction
Multiple myeloma is the second most prevalent hematologic malignancy and is uniformly fatal, very often as a result of development of drug resistance. To overcome the chemoresistance to current therapies and improve patient outcome, novel treatment agents are needed to target mechanisms whereby multiple myeloma cells grow and survive. The coordinated processes of cell-cycle progression, cell growth, and apoptosis are dysfunctional in cancer (1, 2). During cell-cycle progression, microtubule assembly is a proven target for anticancer drug development because of its critical role for mitotic spindle formation and the separation of chromosomes during mitosis (3). It has been shown that the c-jun-NH₂ kinase (JNK)/stress-activated protein kinase (SAPK) pathway is involved in cell-cycle regulation and that microtubule-interfering agents activate this pathway inducing G₂–M arrest that results in apoptosis in a variety of human cancer cells (4).

The Bcl-2 family of proteins includes both pro- and antiapoptotic molecules and their ratio determines the fate of cells. Bcl-2 protein is regulated at transcriptional and posttranslational levels including phosphorylation within the flexible loop regulatory domain. These modifications induce conformational changes in the Bcl-2 protein and regulate its active forms in response to cell death signaling (5, 6). It has been further found that Bcl-2 is phosphorylated/inactivated by JNK/SAPK (7), suggesting that Bcl-2 protein is a target of microtubule-damaging agents, resulting in G₂–M cell-cycle block (8, 9).

Benzimidazoles, including albendazole, fenbendazole, mebendazole, and nocodazole, have been used as anthelmitcits and fungicides on the basis of their antimicrotubule activity (10) and have been reported to elicit promising antitumor effect (11–13). Although nocodazole has been recently categorized as an antineoplastic agent, the antimyeloma effects and its underlying mechanism of action have not been examined yet. By using multiplex cytokine array on a chemical library containing 1,000 compounds provided by the Multiple Myeloma Research Foundation, we identified nocodazole as a potent antimyeloma agent and showed that benzimidazoles, and especially nocodazole, significantly reduce the secretion of cytokines essential for multiple myeloma survival (14).
In the present study, we show that benzimidazoles induce cell death in multiple myeloma cell lines and in primary CD138⁺ myeloma cells and overcome drug resistance. The induction of apoptosis by nocodazole was associated with G₂-M phase of cell-cycle arrest and was induced by JNK-mediated Bcl-2 phosphorylation in both primary and multiple myeloma cell lines. Subsequent induction of apoptosis by nocodazole could be abrogated by a specific JNK inhibitor. Nocodazole, alone or combined with dexamethasone, effectively suppressed myeloma tumor growth in a human multiple myeloma xenograft mouse model, suggesting that nocodazole is a potent and promising new antimyeloma agent.

Materials and Methods

Reagents and cell culture

U0126 and PD98059 [mitogen-activated protein kinase (MAPK) inhibitor], SB203580 (p38-MAPK inhibitor), and SP600125 (JNK inhibitor) were purchased from Calbiochem. Fetal calf serum (FCS) and other cell culture reagents were purchased from Sigma-Aldrich. Albendazole, fenbendazole, mebendazole, and nocodazole (Fig. 1B) were prepared in dimethyl sulfoxide as stock solutions, stored at −20°C, and subsequently diluted in RPMI-1640 medium before use. All drugs were provided by Prestwick Chemicals and obtained within a grant awarded by the Multiple Myeloma Research Foundation.

The following primary antibodies were purchased from Cell Signaling Technology: phospho-c-Jun NH2-terminal kinase, phospho-p38 and phospho-p44/42 MAPKs, phospho-Bcl-2, Bax, Bim, X-linked inhibitor of apoptosis (XIAP), survivin, and Bid. Mcl-1, and death receptor 4 (DR4) antibodies were purchased from Santa Cruz Biotechnology, and actin and β-tubulin conjugated with Alexa Fluor 555 antibodies were purchased from Sigma-Aldrich.

Multiple myeloma cell lines H929 and U266 were purchased from American Type Culture Collection in May 2010 and August 2009, respectively. Dr. William Dalton (H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL) kindly provided the human multiple myeloma cell line RPMI-8226/S and its sublines RPMI-8226/Dox40 (resistant to doxorubicin), RPMI-8226/MR20 (resistant to mitoxantrone), and RPMI-8226/LR5 (resistant to melphalan) in August 2009. Dexamethasone-sensitive and -resistant cell lines MM.1S and MM.1R were provided by Dr. Klaus Podar (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA) in August 2009. These cell lines were tested for their drug resistance, but no cell line authentication was done by the authors. Multiple myeloma cell lines were cultured in RPMI-1640 medium with 10% FCS, 2 mmol/L glutamine, and 100 U/mL penicillin-streptomycin (Sigma-Aldrich) at 37°C with 5% CO₂. The chemoresistant multiple myeloma cell lines were cultured in the presence of doxorubicin, mitoxantrone, melphalan, or dexamethasone, and resistance was confirmed by cell proliferation assays (Supplementary Fig. S1).

Bone marrow mononuclear cells were obtained from untreated multiple myeloma patients and were isolated by separation on Ficoll-Hypaque gradients as described previously (15). CD138⁺ bone marrow cells from multiple myeloma patients were purified by CD138 (syndecan-1) microbeads using a magnetic cell sorting system (Miltenyi Biotec) as described previously (16) and used as primary multiple myeloma cells. Unlabeled cells were used as CD138⁻ cells. All studies were approved by the Institutional Review Board of the University of Pittsburgh, and all subjects signed approved consent forms.

Cell proliferation assay (³H-thymidine incorporation)

U266, H929, RPMI-8226 (3 × 10⁴ cells per well), MM.1S cell line (6 × 10⁴ cells per well), and their resistant sublines were cultured in 96-well culture plates (Costar) in RPMI-1640 medium containing 10% FCS with or without drugs for 48 hours at 37°C with 5% CO₂. For bone marrow stromal cell coculture experiments, human primary bone marrow stromal cells (3 × 10⁵ cells per well) were cultured for 24 hours in Dulbecco’s Modified Eagle’s Medium containing 10% FCS in 96-well plates. Then, multiple myeloma cells (3 × 10⁴ cells per well) were seeded on bone marrow stromal cells and cultured for 48 hours in the presence or absence of nocodazole at different concentrations. Cells were pulsed with 1 μCi/well ³H-thymidine during the last 8 to 10 hours of culture, harvested onto glass fiber filter mats (Wallac) with an automatic cell harvester and counted using a Wallac TriLux Beta plate scintillation counter.

Cell-cycle analyses

After incubation with nocodazole (100 nmol/L for 0–24 hours or 0–160 nmol/L for 16 hours), human multiple myeloma cells were harvested and washed with cold PBS, fixed in 70% ethanol at −20°C, treated with DNase-free RNase A (Sigma-Aldrich), and stained with 50 μg/mL propidium iodide (Sigma) at 37°C for 30 minutes. Analyses were conducted on a BD FACSCalibur flow cytometer and analyzed using ModFit LT 2.0 and Cell Quest software (BD Biosciences).

Assessment of apoptotic cell death and cell viability

Apoptosis was assessed morphologically by evaluation of nuclear condensation and fragmentation using Hoechst 33258 staining as described previously (17). Treated multiple myeloma cells were incubated with 2.5 μg/mL Hoechst 33258 (Molecular Probes, Life Technologies Corp.) for 20 minutes followed by examination under a fluorescence microscope (Olympus CXX41). Cells (~200 per condition) were randomly selected and assessed. Hoechst 33258-positive cells with apoptotic bodies or condensed and fragmented nuclei were considered and counted as apoptotic cells.
Viability of cells was determined by trypan blue staining, which distinguishes the membrane-defective dead cells from the viable cells.

**Immunofluorescence staining and electron microscopy**

Multiple myeloma cells (H929 or RPMI-8226) were fixed in PBS containing 3% formaldehyde for 15 minutes and washed in PBS. Cytospin slides were prepared using Cytospin (Thermo Shandon Inc.) and permeabilized in 0.3% Triton X-100/PBS (30 minutes at 4°C). After blocking with 2.5% bovine serum albumin in PBS, the samples were incubated with Alexa Fluor 555–conjugated antibody against β-tubulin overnight at 4°C. Counterstaining with Hoechst 33258 for nuclear location and integrity was conducted before sealing the slides with Fluoro-Gel (Electron Microscopy Sciences). Slides were examined under a filter-combined fluorescence microscope (Olympus CKX41) equipped with a ×20/0.40 numeric aperture objective lens (Olympus America, Inc.).
For electron microscopy, multiple myeloma cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L PBS (pH = 7.4) followed by 1% OsO4. After dehydration, sections were stained with uranyl acetate and lead citrate and examined under a JEM 1011 (JEOL) electron microscope. To examine and quantify cellular morphologic changes, digital phase-contrast images were recorded.

**SDS-PAGE and Western blotting**

Western blotting was conducted as previously described (18). Briefly, cells were harvested, lysed with radioimmunoprecipitation assay buffer (Pierce) containing phosphatase and protease inhibitors (Halt Protease Inhibitor Cocktail Kit; Pierce). Lysates were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Following probing with specific primary antibodies plus horseradish peroxidase–conjugated secondary antibody, the protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**Human xenograft mouse model**

Beige/nude/X-linked immunodeficient mice were purchased from Charles River Laboratories at 6 to 8 weeks of age with a weight between 20 and 25 grams. For human tumor xenograft studies, 5 × 10⁶ H929 myeloma cells in 50 µL RPMI-1640 together with an equal volume of Matrigel basement membrane matrix (BD Biosciences) were injected subcutaneously as equal volume of Matrigel basement membrane matrix. Animals from both cohorts were euthanized when their tumors reached 2 cm in one diameter and/or were ulcerated (endpoint). Survival was evaluated from the first day of treatment until animals were euthanized.

**Statistical analyses**

All quantitative data are presented as mean ± SD of at least triplicates. Statistical differences were determined by Student’s t test. Kaplan–Meier survival analysis and log-rank test were conducted to estimate the survival rates and survival differences. Results were considered significantly different if P < 0.05.

**Results**

**Nocodazole inhibits myeloma cell growth and overcomes chemoresistance**

We tested the direct effects of benzimidazoles on human multiple myeloma cell proliferation and cell death induction. Albendazole, fenbendazole, and nocodazole significantly inhibited multiple myeloma cell proliferation with an IC₅₀ value of 25 to 94 nmol/L for U266 multiple myeloma cells and an IC₅₀ value of 63 to 380 nmol/L for H929 multiple myeloma cells. Furthermore, benzimidazoles induced nuclear fragmentation, a typical sign of cell apoptosis, with an IC₅₀ value of 30 to 250 nmol/L in RPMI-8226 myeloma cells (Fig. 1A). On the basis of our finding that nocodazole exhibited the strongest antmyeloma effect, we focused our further studies on nocodazole (Fig. 1B, left). Following 48- and 72-hour exposure to nocodazole, the viability of primary CD138⁺ multiple myeloma cells decreased in a dose-dependent manner as monitored by trypan blue staining (IC₅₀ = 125–185 nmol/L). The viability of non–multiple myeloma (CD138⁻) cells obtained from the same patients was affected only at much higher concentration (IC₅₀ ≥ 500 nmol/L), indicating the relative selectivity of nocodazole for malignant plasma cells (Fig. 1B, right).

In addition, the strong inhibition of proliferation of multiple myeloma cells treated with nocodazole was not reversed if multiple myeloma cells were cocultured with bone marrow stromal cells (Fig. 1C).

Because drug resistance is a prevalent problem in multiple myeloma treatment (20), we determined whether nocodazole overcomes drug resistance in multiple myeloma. First, we confirmed the chemoresistant phenotypes of RPMI-8226 sublines (8226/Dox40, 8226/MR20, or 8226/LR5) by culturing the RPMI-8226 cells with doxorubicin, mitoxantrone, or melphalan and analyzing cell proliferation (Supplementary Fig. S1). The drug-resistant sublines exhibited similar responses to nocodazole treatment (IC₅₀ = 25–65 nmol/L) as their parental cell lines. Similar results were observed with dexamethasone-sensitive and -resistant cell lines MM.1S and MM.1R (Fig. 1D), suggesting that nocodazole overcomes chemoresistance.

**Nocodazole-induced prometaphase arrest is associated with disruption of microtubular network array in multiple myeloma cells**

Treatment of H929 multiple myeloma cells with nocodazole resulted in G₂–M phase cell-cycle arrest. After treatment with nocodazole for 7 and 16 hours, the percentage of G₂ population increased to 50% and 35%, respectively, in comparison with control (21%). Prolonged treatment was associated with increased...
apoptosis of multiple myeloma cells, as evidenced by the increased population of pro-G0 phase cells as well as the appearance of polyploid cells (Fig. 2A, top). In accordance with the time-dependent induction of G2 arrest, we also observed a dose-dependent G2 arrest with an increase of G2 population to 40% at 80 nmol/L and 56% at 160 nmol/L in comparison with control (19%; Fig. 2A, bottom).

To identify mitotic phase arrest in nocodazole-treated multiple myeloma cells, we examined microtubule dynamic change and chromosome status as well as nuclear membrane integrity. Immunofluorescence staining of the vehicle-treated cells for tubulin showed the regular structure of microtubules with cells in anaphase/telophase. However, nocodazole-treated multiple myeloma cells displayed a disordered microtubule structure. Spindle formation was disrupted and cells exhibited an irregular staining pattern for tubulin along with some small punctate areas (Fig. 2B). The mitosis-like chromosomes with disordered orientation were condensed and scattered inside the cells. The peak of destruction of the microtubular network was observed after 16 hours of nocodazole treatment (Fig. 2B and Supplementary Fig. S2A). Electron microscopy studies indicated that the nuclear membrane had collapsed and disappeared (Fig. 2C), which is a typical phenomenon of prophase and prometaphase, suggesting that the cells arrested in prophase/prometaphase.

We found that nocodazole also caused significant (P < 0.05) morphologic elongation of multiple myeloma cells (Fig. 2B and C and Supplementary Fig. S2A–C). Most of the elongated cells only had one irregular nucleus.
consisting of clusters of condensed chromosomes, suggesting that the cells failed to progress into mitosis. Importantly, immunofluorescence staining for tubulin showed that the disordered structure of microtubules was only observed in the elongated cells (Fig. 2B and Supplementary Fig. S2A). These data suggest that nocodazole induces damage of the microtubular network and disrupts the interaction of microtubules and kinetochore, resulting in the prevention of the formation of the mitotic spindle and the arrest in mitosis prophase/prometaphase.

JNK-mediated Bcl-2 phosphorylation contributes to nocodazole-induced cell death

To study the molecular mechanism underlying nocodazole-induced myeloma cell death, we focused on the apoptosis-regulatory protein Bcl-2. Bcl-2 has been suggested to regulate microtubule integrity (21) and is rapidly phosphorylated and inactivated in response to exposure of cells to microtubular disrupting agents. Bcl-2 phosphorylation was strongly induced in a dose- and time-dependent manner at both Ser70 and Thr56 sites whereas total Bcl-2 protein was unaffected by nocodazole treatment in myeloma cells (Fig. 3A–C). Importantly, upregulation of Bcl-2 phosphorylation and proapoptotic BH3-only protein Bim isoforms (BimEL, BimL, and BimS) was also observed when primary CD138\(^+\) myeloma cells were treated with nocodazole (Fig. 3B and C, left). On the contrary, nocodazole exposure had no significant effect on the Bcl-2 phosphorylation and Bim isoforms in nonmyeloma CD138\(^-\) cells (Fig. 3B and C, right). Nocodazole decreased the protein levels of antiapoptotic XIAP and Mcl-1\(_L\) but had no effect on proapoptotic molecules such as Bid and Bax as well as death receptor 4 and the apoptosis inhibitor, survivin (Fig. 3A and D).

Several protein kinases have been identified to be critical for Bcl-2 phosphorylation (5). We found that nocodazole phosphorylates JNK and p38-MAPK and decreases the phosphorylation of the extracellular signal–regulated kinases (ERK) in a dose- and time-dependent manner (Fig. 4A). JNKs, in contrast to p38-MAPK or ERKs, contribute primarily to the induction of Bcl-2 phosphorylation. To confirm whether only JNK induces phosphorylation of Bcl-2 protein, we treated RPMI-8226 multiple myeloma cells with nocodazole in the presence of specific MAPK inhibitors. Inhibition of MEK-ERK (UO126 and PD98059) and p38-MAPK (SB202190) pathways had no effect on the phosphorylation of Bcl-2 protein. Only the JNK inhibitor (SP600125) was able to abrogate Bcl-2 phosphorylation at both Ser70 and Thr56 sites (Fig. 4B). In accordance with this is the finding that only JNK inhibitor SP600125 prevented nocodazole-induced cell nuclear fragmentation. Compared with vehicle control, nocodazole-induced cell death was significantly decreased by 50% and 64% when JNK pathway was blocked by...
SP600125 at 10 µmol/L and 20 µmol/L, respectively. Other MAPK inhibitors failed to rescue multiple myeloma cells from nocodazole-induced cell death (Fig. 4C).

A high amount of phosphorylated Bcl-2 protein was detected specifically in G2–M phase within 16 hours of nocodazole exposure (Figs. 2A and 3A). These results in combination with the known role of Bcl-2 phosphorylation in all cell cycles indicate that nocodazole-induced Bcl-2 phosphorylation is associated with accumulation and arrest of cells in G2–M phase that can be abrogated by JNK inhibitor treatment.

**Nocodazole alone and in combination with dexamethasone inhibits multiple myeloma tumor growth in vitro and in vivo**

To further investigate the effects of nocodazole, we tested nocodazole in combination with other compounds. As shown in Fig. 5A, combination of nocodazole with lenalidomide, dexamethasone, or a novel histone deacetylase inhibitor inhibitor KD5170 (17) resulted in a significant (P < 0.05) inhibition of proliferation compared with either drug alone. Analysis of nuclear fragmentation as a marker for cell death indicated that dexamethasone (20 nmol/L) alone induced less than 20% cell death. Low concentrations (15 and 30 nmol/L) of nocodazole alone induced 10% and 16% nuclear fragmentation. However, combination of 20 nmol/L dexamethasone with 15 or 30 nmol/L nocodazole significantly increased the percentage of apoptotic cells to 67% and 92%, respectively (P < 0.01; Fig. 5B).

To assess the antamyeloma effect of nocodazole in vivo, we used the human xenograft mouse model using human multiple myeloma cells (H929). H929 multiple myeloma cells grow vigorously and behave as an aggressive tumor in vivo. To titrate a treatment dosage, we used high (20 mg/kg) and low (5 mg/kg) concentrations of nocodazole without dexamethasone. Nocodazole (20 mg/kg) injected intraperitoneally 3 times weekly showed significant inhibition of tumor growth as early as the tenth day of treatment (P < 0.02) compared with that of the control group (Supplementary Fig. S3). At 20 mg/kg nocodazole, no severe side effects, such as weight loss or diarrhea, were observed but the mice showed mild dry skin with scales. At 5 mg/kg, mice showed no physical changes. For further in vivo testing, an intermediate dose of 12 mg/kg nocodazole was chosen. Because our in vitro data showed that combination of nocodazole and dexamethasone significantly enhances apoptosis of multiple myeloma cells (Fig. 5B), combination of nocodazole and dexamethasone was tested in the abovementioned mouse model. Mice were treated with an intermediate dose of nocodazole (12 mg/kg intraperitoneally 3 times weekly),...
the treatment period for all the treatment groups as shown in Fig. 6B). No adverse toxicity was observed throughout the experiment (Fig. 6A). Kaplan–Meier survival analysis indicated that the combination treatment significantly prolonged survival time in comparison with the non- or slow-cycling controls and of multiple myeloma cell lines alone and in coculture with bone marrow stromal cells by inducing cell-cycle arrest and subsequent apoptosis. Inhibition of cell proliferation by nocodazole occurred independent from MDR of the multiple myeloma cell lines, suggesting that nocodazole overcomes cell adhesion–mediated resistance and MDR to conventional therapies. In addition, its relative selective cytotoxicity to multiple myeloma cell lines and primary myeloma cells, but not to nonmalignant bone marrow mononuclear cells, suggests a favorable therapeutic index. Our in vitro data on combination studies show that nocodazole with compounds, such as dexamethasone, significantly increases the antimyeloma effect (Fig. 5B), but further studies are needed to explore additive or even synergistic effect. In vivo nocodazole combined with dexamethasone significantly inhibited tumor growth and prolonged survival in a human xenograft myeloma mouse model. Furthermore, our data show that in cycling cells, nocodazole affects the microtubule assembly and causes mitotic arrest, which in turn leads to apoptosis. The higher selectivity of nocodazole toward tumor cells might result from targeting cycling cells compared with the non- or slow-cycling cells. At prometaphase, the phase of mitosis following prophase and preceding metaphase, the nuclear envelope fragments and disappears. The role of prometaphase is completed when all of the microtubules have attached to their kinetochores, at which point metaphase begins (27). In nocodazole-treated multiple myeloma cells, the antimyeloma activity of nocodazole was observed as early as day 8 that persisted for the entire experiment (Fig. 6A). Kaplan–Meier survival analysis indicated that the combination treatment significantly prolonged survival time in comparison with the non- or slow-cycling controls and of multiple myeloma cell lines alone and in coculture with bone marrow stromal cells by inducing cell-cycle arrest and subsequent apoptosis. Inhibition of cell proliferation by nocodazole occurred independent from MDR of the multiple myeloma cell lines, suggesting that nocodazole overcomes cell adhesion–mediated resistance and MDR to conventional therapies. In addition, its relative selective cytotoxicity to multiple myeloma cell lines and primary myeloma cells, but not to nonmalignant bone marrow mononuclear cells, suggests a favorable therapeutic index. Our in vitro data on combination studies show that nocodazole with compounds, such as dexamethasone, significantly increases the antimyeloma effect (Fig. 5B), but further studies are needed to explore additive or even synergistic effect. In vivo nocodazole combined with dexamethasone significantly inhibited tumor growth and prolonged survival in a human xenograft myeloma mouse model. Furthermore, our data show that in cycling cells, nocodazole affects the microtubule assembly and causes mitotic arrest, which in turn leads to apoptosis. The higher selectivity of nocodazole toward tumor cells might result from targeting cycling cells compared with the non- or slow-cycling cells. At prometaphase, the phase of mitosis following prophase and preceding metaphase, the nuclear envelope fragments and disappears. The role of prometaphase is completed when all of the microtubules have attached to their kinetochores, at which point metaphase begins (27). In nocodazole-treated multiple myeloma cells, the
molecular cancer therapeutics

Molecular Cancer Therapeutics

Mcl-1L downregulation by nocodazole indicates a link to apoptosis. The functional significance of XIAP and work damage leads to the release of Bim and apoptosis indicating that nocodazole-induced microtubular network damage (35). This is in accordance with our data that cells treated with nocodazole arrest with G2 or M phase DNA content, nocodazole is frequently used to synchronize the cell division cycle (24, 31–33), and our findings of myeloma cell death after prolonged arrest of cells in mitotic prometaphase is not unexpected (34).

Further analysis of prodeath BH3-only molecules showed that Bim isoform proteins were increased in both myeloma cell lines and primary CD138+ cells. Bim is usually sequestered in the cytosol by binding with the dynein light chain-1 and is released from the microtubules in response to apoptotic stimuli via microtubular damage (35). This is in accordance with our data indicating that nocodazole-induced microtubular network damage leads to the release of Bim and apoptosis induction. The functional significance of XIAP and Mcl-1 downregulation by nocodazole indicates a link between cell-cycle arrest and cell apoptosis (36, 37). Furthermore, downregulating XIAP has been shown to promote persistent JNK activation (38), which may induce Bcl-2 phosphorylation. Our data show that nocodazole-induced death of myeloma cells is associated with JNK activation and Bcl-2 phosphorylation. This is in accordance with data showing that activation of JNK by drug treatment induces Bcl-2 phosphorylation on specific residues including Ser70, which leads to Bcl-2 inactivation and apoptosis (6, 9, 21). Furthermore, it was also suggested that Bcl-2 phosphorylation might represent a preapoptotic phase after microtubule damage and subsequently dephosphorylation initiates apoptosis (39). We found that phosphorylation of Bcl-2 at Ser70, and to a less degree at Thr56, was markedly induced after treatment with nocodazole. Specific inhibition of JNK1/2 prevented nocodazole-induced Bcl-2 phosphorylation (Fig. 4B). In accordance with this, nocodazole-induced myeloma cell apoptosis was also reduced by concomitant treatment with JNK1/2 inhibitor (Fig. 4C). The connection of JNK activation and Bcl-2 phosphorylation was further shown by comparing the overlap between kinetics and dose dependence of these kinase-driven events (Figs. 3 and 4A). Furthermore, it has been shown that JNK-induced Bcl-2 phosphorylation diminished the binding activity of Bcl-2 to both multidomain and BH3-only proapoptotic family members (40, 41), thereby facilitating the proapoptotic activity of these members. Therefore, our results are consistent with findings from other investigators showing that the JNK pathway is responsible for phosphorylation of Bcl-2 in the same loop region in response to microtubule-damaging agents (42, 43).

In summary, our studies show that nocodazole targets the multiple myeloma cell and its microenvironment (14). Nocodazole mediates its antmyeloma activity through sequential microtubular network damage and cell-cycle arrest. JNK-mediated Bcl-2 phosphorylation results in multiple myeloma cell apoptosis. Nocodazole overcomes drug resistance, decreases tumor growth, and extends survival in vivo in human xenograft mice model. The known toxicity profile and selective activity against myeloma cells provide the rationale for considering nocodazole as future treatment for multiple myeloma.

Disclosure of Potential Conflicts of Interest

There are no conflicts of interest to disclose.

Figure 6. Nocodazole significantly inhibits tumor growth and prolongs survival in the human xenograft mouse model. H929 multiple myeloma cells mixed with Matrigel were injected subcutaneously into the right flank of beige/nude/X-linked immunodeficient mice. A, once tumors became palpable, mice were treated (intraperitoneally) with vehicle solution, dexamethasone alone (Dex, 2 mg/kg), nocodazole (Noco, 12 mg/kg) alone, or the combination of dexamethasone and nocodazole (n = 5 each group). Results are shown as mean ± SD of tumor volume. *, P < 0.02; **, P < 0.01 compared with vehicle-treated mice. B, survival rate was evaluated using Kaplan–Meier method and log-rank analysis. Combination group (Noco + Dex) showed significantly increased survival (P < 0.01) compared with control.
Antimyeloma Activity of Nocodazole

Authors' Contributions

R. Feng designed and carried out experiments, analyzed data, and wrote the manuscript; S. Li and C. Lu carried out experiments; C. Andreas conducted bone marrow collection; D.B. Solz assisted in electron microscopy studies; M.V. Mapara analyzed data and reviewed the manuscript; and S. Lentzsch designed the experiments, analyzed data, and wrote the manuscript.

Acknowledgments

We would like to thank Dr. Jie Han and E. Michael Meyer for assistance with flow cytometric studies. The authors thank Dr. William Dalton (H. Lee Moffitt Cancer Center & Research Institute, University of South Florida) for providing the human multiple myeloma cell line RPMI-8226/S and its sublines RPMI-8226/DoxR, RPMI-8226/MR20, and RPMI-8226/LR5. Desamethasone-sensitive and -resistant cell lines MML.1S and MML.1R were obtained from Dr. Klaus Pudar (Dana Farber Cancer Institute, Harvard Medical School). We thank Ms. Rita Bhutta for excellent preparation of the manuscript.

Grant Support

The current study was supported in part by research funding from Multiple Myeloma Research Foundation to S. Lentzsch.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 29, 2011; revised July 12, 2011; accepted August 1, 2011; published OnlineFirst August 8, 2011.

References

Molecular Cancer Therapeutics

Targeting the Microtubular Network as a New Antimyeloma Strategy
Rentian Feng, Shirong Li, Caisheng Lu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0234

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2011/08/08/1535-7163.MCT-11-0234.DC1

Cited articles
This article cites 43 articles, 27 of which you can access for free at:
http://mct.aacrjournals.org/content/10/10/1886.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/10/10/1886.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.